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(54) Title: RECOMBINANT EXPRESSION SYSTEM BASED ON SATELLITE TOBACCO MOSAIC VIRUS

(57) Abstract

　　The present invention provides the sequence of the RNA genome of satellite tobacco mosaic virus. Based on said sequence and the discovery that heterologous RNA can be accommodated in said genome without eliminating replicability, the invention provides compositions comprising modified genomes of the virus, or cDNAs of such genomes, to transform plant cells in vitro and in vivo to make desired RNAs or proteins. Further, the invention provides methods and intermediates for making such compositions and methods of using such compositions.

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RECOMBINANT EXPRESSION SYSTEM BASED ON
SATELLITE TOBACCO MOSAIC VIRUS

TECHNICAL FIELD

5   Th present invention relates to genetic
engineering of plants and, more particularly, to such
engineering utilizing the properties of RNA plant
viruses.

        Thus, the invention concerns satellite
10 tobacco mosaic virus (STMV), recombinant STMV RNA
molecules containing exogenous RNA segments, which are
heterologous to naturally occurring STMV RNAs, and a
recombinant expression system making possible production
of a desired gene product in the cytoplasm of a plant
15 infected with recombinant STMV RNA molecules and a helper
virus.

        In one aspect, the present invention provides
infectious recombinant RNA molecules derived from
satellite tobacco mosaic virus (STMV) ssRNA.
20   In another aspect, the present invention
concerns DNA transcription vectors containing
substantially full-length cDNA copies of infectious STMV
ssRNA.

        In a further aspect, the invention relates to
25 infectious recombinant RNA molecules derived from STMV
ssRNA, having an exogenous RNA segment at a site that is
non-essential for RNA replication in a host cell.
        In a still further aspect, the present
invention relates to DNA transcription vectors containing
30 substantially full-length cDNA copies of such infectious
recombinant RNA molecules.

        The present invention further concerns a method
of transforming plant cells by introducing into the
cytoplasm of such cells infectious recombinant RNA
35 molecules derived from STMV ssRNA, having such exogenous
RNA segments, and a helper virus of STMV.

        The present invention also provides a method
for the production of exogenous proteins in the cytoplasm

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of plant cells by utilizing such genetically manipulated
infectious RNA mol cul s, or corresponding cDNAs, and a
helper virus of STMV.

5   BACKGROUND OF THE INVENTION
            Members of several different plant virus groups
support the replication of another virus (Kassanis,
Intervirology 15, 57-70 (1981); Buzen et al.,
Phytopathology 74, 313-8 (1984); Gingery and Louie,
10   Phytopathology 75, 870-4 (1985)). Viruses which have a
dependence on another virus for their replication were
first reported by Kassanis, J. Gen. Microbiol. 27, 477-
488 (1962) and are known as satellite viruses. Satellite
viruses of plant viruses depend completely on a specific
15   helper virus (or viruses) for their replication but
appear to share little, if any, nucleotide sequence
similarity with their helpers (Francki, Ann. Rev.
Microbiol. 39, 151-74 (1985); Murant and Mayo, Ann. Rev.
Phytopathol. 20, 49-70 (1982)). Satellites may interfere
20   with helper virus synthesis or may modify disease symptom
expression in the host plant. Satellite viruses differ
from so-called satellite RNAs. A satellite virus encodes
a capsid protein that specifically encapsidates its own
satellite virus RNA. In contrast, a satellite RNA is
25   encapsidated by capsid protein provided by an helper
virus RNA.
            Satellite viruses include those of tobacco
necrosis virus ((STNV); Kassanis, Intervirology 15, 57-70
(1981)), panicum mosaic virus ((SPMV); Buzen et al.,
30   Phytopathology 74, 313-8 (1984); Masuta et al., Virology
159, 329-38 (1987)) and maize white line mosaic virus
(Gingery and Louie, Phytopathology 75, 870-4 (1985)).
The helper viruses of these satellite viruses are
unrelated, but all of the helpers have monopartite
35   single-stranded RNA (ssRNA) genomes and exist as
icosahedral 30nm particles. The satellite viruses all
exist as 17nm icosahedral particles which are

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antigenically unr lated to their h lp rs and to each
other. Although STNV and SPMV have been well
characterized and the nucleotide s quences of their
genomes have been determined, there are no data on the
5 genome sequences and genome organizations of their
respective helpers.

Satellite tobacco mosaic virus (STMV) is the
most recently identified plant satellite virus (Valverde
and Dodds, J. Gen. Virology 67, 1875 (1986) and J. Gen.
10 Virology 68, 965 (1987)). It consists of a single,
0.3 x 10⁶ Mr linear, messenger-sense, single-stranded RNA
(ssRNA) and multiple copies of a 17,500 Mr capsid protein
and depends on helper viruses for its replication. All
of STMV's helper viruses are rod-shaped tobamoviruses,
15 including tobacco mosaic virus (TMV), that is a prototype
member of the group.

The structure of STMV, including the complete
nucleotide sequence of its ssRNA genome, has been
thoroughly investigated (E.Mirkov: cDNA Cloning,
20 Nucleotide Sequences, in vitro Translation, and Genome
Organization of STMV, Dissertation, University of
California, Riverside (March 1988)). However, as will be
described more specifically hereinafter, the published
sequence of the STMV RNA contains numerous significant
25 errors, particularly in the 3'- and 5'-termini, and the
total length of the ssRNA genome as reported (1,065
ribonucleotides) is incorrect. The STMV ssRNA has two
open-reading frames (ORFs). The first ORF encodes a
6,800 Mr protein that corresponds in size to a major in
30 vitro translation product directed by STMV RNA. The
second ORF encodes a 17,500 Mr protein that corresponds
in size to the other major in vitro translation product
synthesized under the direction of STMV RNA. The first
12 codons of this second ORF were found to correspond to
35 the sequence of 12 N-terminal amino acids of the capsid
protein. Western-blot analysis of these in vitro
translation products reveal d that the 17,500 Mr protein

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is antigenically related to the authentic capsid protein,
while the 6,800 Mr protein is not. Time course analysis
of _in vitro_ translation products d monstrated that the
6,800 Mr protein is synthesized at the same time as the
5   capsid protein and does not, therefore, arise by the
proteolytic cleavage of a larger precursor polypeptide,
suggesting that the genome of STMV acts as a
polycistronic, eukaryotic, messenger RNA.

        The tobamoviruses, to which the known helper
10   viruses of STMV belong, are known in, and available to,
the art. Tobacco mosaic virus (TMV), for example, is one
of the best characterized plant viruses from molecular
biological and physicochemical points of view. For a
review of this group see _Molecular Plant Virology_,
15   Volume II, J. W. Davies, editor, CRC Press Inc., Boca
Raton, Florida U.S.A. (1985). Known representatives of
this group include the TMV strains U1, U2 and U5. These
strains are described in Goelet _et al._, _Proc. Natl. Acad._
Sci. USA _78_, 5818 (1982) and Garcia-Arenal, _Virology_ _166_,
20   201 (1988) and infectious clones of the U1 strain are
available from Prof. William Dawson, University of
California, Riverside.

        The preparation of infectious RNA transcripts
from cloned cDNAs of cucumber mosaic viral satellites is
25   described in Whitmer et al., _Biochemical and Biophysical_
Research Communications _135_, 290 (1986). Complete cDNA
copies of two variants of the satellite of cucumber
mosaic virus, CARNA 5, were cloned in a transcription
vector, and coinfected with cucumber mosaic viral RNAs on
30   tomato plants.

        Meshi et al., _Proc. Natl. Acad. Sci. USA_ _83_,
5043 (1986) cloned full-length double-stranded cDNAs of
tobacco mosaic virus (TMV) RNA into a transcription
vector, and the _in vitro_ RNA transcripts were used to
35   infect tobacco plants.

        Emmelo et al., _Vir logy_ _157_, 480 (1987)
reported that mechanical inoculation of cowpea leaves

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with cloned full-size copies of satellit tobacco
n crosis virus (STNV) in the presence of its helper,
tobacco necrosis virus (TNV), r sulted in the appearance
of infectious virus particles.

5   In vitro synthesis of infectious RNA copies of
the virulent satellite (RNA C) of turnip crinkle virus
(TCV) is disclosed in Simon et al., Virology 156, 146
(1987). Full-length cDNA copies of the satellite were
inserted into an expression vector, and the RNA
10   transcripts synthesized in vitro from the cDNAs were
coinoculated with helper virus RNA into 3-week-old
turnips. Plus-strand RNA copies of the satellite were
found to be infectious, while minus-strand copies were
not.

15   European Patent Application Publication
No. 0 248 077 discloses a process for increasing protein
production from a cDNA encoding an eukaryotic protein by
joining to the 5'-terminus of the cDNA a nucleotide
sequence which has a regulatory role, in expression of a
20   coat protein of a plant virus, by increasing competitive
activity and RNA translation leading to the coat protein.
Suitable viruses as sources of the nucleotide sequence,
which regulates expression of coat protein, are said in
the published application to include alfalfa mosaic
25   virus, brome mosaic virus, black beetle virus, turnip
yellow mosaic virus, and satellite tobacco necrosis
virus.

          European Patent Application Publication
No. 0 194 809 discloses the modification of the genome of
30   brome mosaic virus (BMV) to include an exogenous (i.e.,
heterologous) RNA segment, and the introduction of the
modified RNA into a host cell, wherein the virus
replicates and an exogenous gene product (i.e., a gene
product heterologous to BMV) is produced. BMV is a
35   multipartite RNA virus, i.e., the total genetic
information required for replication and productive
infection is divided into more than one discrete RNA

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molecule. According to EPA 0 194 809, the cDNA
corresponding to one of the RNA components (RNA3) of the
BMV genome was modified by replacing a certain area (the
RNA4 coding sequence) with a CAT gene (i.e., a cDNA
5   encoding bacterial chloramphenicol acetyltransferase
(CAT)). From the cDNAs corresponding to the RNA
components of the virus genome, including the modified
cDNA, transcripts were made, and barley seedlings were
infected with a mix of the cDNA transcription products.
10   The fact that the RNA4 coding sequence of the RNA3
component of BMV is not required for infectivity was
disclosed by Lane et al., Nature 232, 40 (1971). In
mixing experiments, the authors found that infectivity
resulted when the combination of RNAs 1, 2, and 3 was
15   used, and "the omission of any except component 4 from a
mixture markedly decreases specific infectivity".
        European Patent Application Publication
No. 0,229,174 describes production of transgenic plants,
that are able to express foreign proteins, by infection
20   with a retroviral vector.
        An expression system for making desired
proteins in plants and based on satellite tobacco mosaic
virus (STMV) has heretofore not been available or known.
The structure and properties of STMV, including those
25   which the present inventors have discovered as described
hereinbelow, make the virus particularly apt as a target
for genomic modification and associated use to prepare
vectors for such an expression system. Until the present
invention, such use of the virus and the advantages of
30   such use could not be realized.

SUMMARY OF THE INVENTION
        The present invention provides, for the first
time, the correct complete nucleotide sequence of the
35   genomic RNA of satellite tobacco mosaic virus (STMV).
The sequence as earlier published (see Mirkov's
Diss rtation, supra) contained several incorrect

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nucleotides at and near the 3'- and 5'-termini, and
erroneously included extra nucleotides that resulted in a
longer RNA molecule (1,065 nucleotides) than the viral
RNA (1059 nucleotides) actually has. It has been found
that the 3'-terminal nucleotides of STMV RNA and either
TMV U1 or TMV U2/U5 show a great degree of sequence
similarity, with two nearly identical regions of 40 and
50 bases, respectively. This is surprising in view of
the earlier assumption that satellite viruses and their
helpers share little, if any, genomic sequence
similarity.

The invention further provides infectious
recombinant RNA molecules that are derived from STMV
ssRNA, preferably via transcription from a substantially
full-length cDNA copy of the genomic STMV ssRNA or a
modification thereof which incorporates a segment coding
for an exogenous protein (i.e., a protein heterologous to
those made by the naturally occurring STMV genome)
desired to be expressed in a plant cell infected with the
ssRNA molecules.

The present invention further relates to a
recombinant expression system based on the use of STMV.
The expression system allows for expression of a desired
exogenous gene (i.e., one heterologous to the naturally
occurring STMV genome), in the cytoplasm of a plant
coinfected with recombinant STMV RNA molecules of the
invention and a helper virus. Because the infection
remains cytoplasmic, the recombinant trait is not passed
on to progeny plants and is thus easily contained in the
plant. The expression system can, for example, be used
to transfer economically attractive traits, e.g. disease
resistance, to plants.

The expression system of the present invention
provides the following major advantages over the already
known systems:

1. The genome of STMV encodes a demonstrable _in
vivo_ protein product, a coat protein, which is not the

case for some oth r currently popular model systems, such
as the systems based on the satellite cucumber mosaic
virus and tobacco ringspot virus. This allows for an
easy assay of gene expression.

5   2. In contrast to satellite tobacco necrosis
virus (STNV), which also encodes its coat protein, STMV
is systemically distributed in the host in which it
accumulates, as is its helper. This means that
expression of a foreign gene in the host may well be
10   throughout an entire plant.

           3. The plant hosts used when working with this
satellite are herbaceous, including many solanaceous
plants, for which transformation systems are available.

           4. The STMV virus is readily introduced into
15   plants as virion particles or RNA by mechanical
inoculation, and activation is with a helper virus, which
is also mechanically transmitted.

           5. The spherical particles of the satellite are
readily distinguished from the rod-shaped particles of
20   its helper.

           6. The genome structure is unique when compared
to other satellite viruses. These unique features
include non-phosphorylated 5'-termini, the degree of
similarity to its helper viruses, the genome
25   organization, and the ability to function as a
polycistronic mRNA.

           In one aspect, the present invention relates to
an infectious recombinant RNA molecule derived from
satellite tobacco mosaic virus (STMV) ssRNA. Preferably,
30   said RNA molecule is unaccompanied by endogenous
components not essential for its infectious properties,
and is preferably derived from an authentic STMV ssRNA
molecule, having a nucleotide sequence as shown in
Figure 1.

35   In a further aspect, the invention concerns a
DNA transcription vector containing a substantially full-
length cDNA copy of an infectious STMV ssRNA.

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In a still further aspect, th present
invention r lates to infectious rec mbinant RNA molecules
derived from STMV ssRNA, having an xog nous RNA segment
at a site that is non-essential for RNA replication in a
5   host cell. Preferably, this exogenous RNA segment is
located within the open reading frame encoding the STMV
coat protein.

The present invention further relates to DNA
transcription vectors containing a substantially full-
10   length cDNA copy of such infectious recombinant RNA
molecules.

In another aspect, the present invention con-
cerns a method of transforming plant cells, comprising
introducing into the cytoplasm of such cells a recom-
15   binant RNA molecule derived from STMV ssRNA, having an
exogenous RNA segment at a site that is non-essential for
RNA replication in such host cells, and either (a)
infecting the cell with an helper virus of STMV or (b)
otherwise introducing into the cell the genome of an
20   helper virus of STMV. As such, the invention also
encompasses a composition which comprises in combination,
in a plant cell or in a mixture employed in transforming,
or employable to transform, a plant cell, such a
recombinant RNA molecule and either an helper virus of
25   STMV or the genome of an helper virus of STMV.

In a still further aspect, the present
invention concerns a method for the production of an
exogenous protein in the cytoplasm of a plant cell, which
has been co-infected with an infectious recombinant STMV
30   RNA molecule as hereinabove described, or with a cDNA
having one strand substantially complementary to said RNA
and capable of providing said recombinant RNA _in vivo_,
and either (a) an helper virus of STMV or (b) (i) the
genome of an helper virus of STMV or (ii) a cDNA having
35   one strand substantially complementary to the genome of
such an helper virus and being capable of providing said
genom _in vivo_ in the cell, which method comprises

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exposing said c -infected plant cell to, or maintaining
said co-infected bplant cell under, c nditions whereby
said protein is made in th cell by translation of said
infectious recominnant STMV RNA.

5   It is further possible to introduce infectious
recombinant STMV RNA or cDNA molecules into plants,
following plant transformation with Agrobacterium
tumefaciens carrying modified, recombinant Ti plasmids.
Since the expression of the exogenous sequences present
10   in the recombinant STMV RNA molecules is dependent upon
infection with an helper virus such as TMV, the helper
virus can be used as a regulatory trigger for the
expression of the gene(s) foreign to the STMV genome. A
particular use for such a system is the introduction of
15   presumed cross-protection genes from other viruses, such
as cucumber mosaic virus, into plants susceptible to TMV,
and high-level expression of antisense RNA for control of
plant viral diseases. This particular aspect is also
within the scope of the invention.

20   The present invention is directed to the above
aspects and all associated methods and means for
accomplishing such. For example, the invention includes
the technology requisite for isolation and purification
of STMV ssRNA, transcription procedures, methods for
25   sequencing genomic RNA, cDNAs and RNA transcripts, plant
inoculation techniques, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

             Figure 1 depicts the nucleotide sequence
30   (genomic sense) of STMV RNA and the deduced amino acid
sequences of the two open-reading frames. Stop codons
are identified as *. Regions of the coat protein from
which amino acid sequence data were obtained are
underlined.

35   Figure 2A is a sequence comparison of the 3'-
terminal regi ns of STMV RNA (middl line of each
comparison, bas s 770 to 1059), TMV U2/U5 RNA (upper line

of each comparison, 110 terminal 3' nucleotides), and TMV
U1 RNA (lower line of each comparison, bases 6100 to
6395). Identity is indicated by a "*" and gaps generated
to provide a best fit are indicated with "----". The
5   stop codon for the coat protein is underlined for TMV U1
and U5.

Figure 2B is a comparison of the 5' sequences
of STMV RNA, BMV-RNA3 and CMVQ-RNA3.

Figure 2C is a comparison of the 5' sequences
10   of STMV RNA, STNV RNA and SPMV RNA.

Figure 3 shows the strategy used towards
construction of a complete genomic clone for STMV. Clone
pSTMV-2 (designated "2" in the Figure) was joined to
clone pSTMV-13 (designated "13" in the Figure) at a
15   unique DraI site to produce clone pSTMV2+13. A near
full-length clone was created by digestion of double-
stranded cDNA of STMV with BglII and HindIII to yield a
fragment (designated "31", for clone pSTMV-31, in the
Figure) which was ligated to pSTMV2+13 that had been
20   digested with BglII and HindIII. The resulting
construct, pSTMV3, shown at the bottom of the Figure,
lacks only the 73 5' terminal bases. The heavy line at
the top of the figure is a partial restriction map of
STMV cDNA.

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DETAILED DESCRIPTION OF THE INVENTION
Definitions

The term "infectious" used in connection with
RNA or cDNA molecules derived from STMV ssRNA indicates
30   that the STMV viruses having such RNA, per se or made
from such a cDNA molecule, in their genome are capable of
replication and accumulation in living cells. Such
molecules do not contain any sequence, either as a result
of spontaneous mutation or as a consequence of genetic
35   manipulation, that would disrupt virus RNA replication.

The term "derived from" is used to refer to a
viral RNA, typically one that is naturally occurring,

that is modified in a recombinant RNA molecule according
to the invention. Recombinant RNAs described herein are
d rivativ s of STMV genomic RNAs. Substantial portions
(i.e., segments) of these recombinant RNAs are from STMV
5   genomic RNA. However, components that are endogenous to
STMV genomic RNA but are not essential for infectivity
may be omitted, or replaced, for example by exogenous RNA
segments, in the recombinant RNAs according to the
invention that are derived from STMV genomic RNA. In
10   addition, natural mutations or induced changes in the
STMV ssRNA segments, which do not eliminate the
biological activities of the segements or, if they result
in amino acid changes in encoded proteins, do not
eliminate the biological activites of the proteins, may
15   be present. For deriving the recombinant RNA molecules
according to the present invention from STMV ssRNA,
different methods may be employed. The manner of
deriving may, for example, be by transcription, direct
recombination at the RNA level, or insertion of
20   heterologous DNA segments into cDNA copies of the STMV
genomic RNA followed by transcription of the cDNA copies.

The terms "capsid protein" and "coat protein"
are used interchangeably and refer to a 17,500 Mr protein
encoded by the open reading frame beginning at nucleotide
25   163 in the authentic nucleotide sequence of STMV ssRNA
(see Fig. 1).

The term "substantially full-length copy of an
infectious STMV ssRNA" is used to refer to cDNA molecules
that may not be exact copies of the respective infectious
30   ssRNAs but comprise the complement of every nucleotide
that is required for infectivity. Such cDNA molecules
may be shorter or longer than the ssRNA molecules they
complement, and even may include nucleotides with no
corresponding ribonucleotide in the RNA, as long as the
35   infectivity is preserved.

The term "degenerate equivalent thereof," as
used in connection with STMV ssRNA, and RNA of the

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inventi n, or a substantially full-length DNA copy of
either, means an equivalent which differs in nucleotide
sequence but ncod s the same amino acid s quence(s),
taking account of the degeneracy of the genetic code.
5   Thus, a multitude of different nucleic acid sequences
encoding the same amino acid sequence are considered
degenerate equivalents as defined herein. In addition,
natural mutations or induced changes in the RNA or DNA
segments, which do not eliminate the biological
10   activities of the segments or, if they result in amino
acid changes in encoded proteins, do not eliminate the
biological activites of the proteins, fall within the
scope hereof and are considered equivalents as well.

        The term "exogenous RNA segment," as used
15   herein, refers to an RNA segment which, in an infectious
RNA according to the invention, is heterologous to
naturally occurring STMV ssRNA, e.g., a segment which
does not occur in such STMV ssRNA. Typically, the
exogenous RNA segment will be a segment inserted into the
20   segment of STMV RNA encoding the coat protein or
replacing all or part of such coat-protein-encoding RNA.
The exogenous RNA segment will typically itself encode
some desired protein. The source of an exogenous RNA
segment may be a natural source, including viruses other
25   than STMV, bacteria, yeasts, fungi, plants and animals;
or the exogenous RNA segment may be entirely or partly
synthesized. The exogenous RNA fragments may have
different functions, including, but not limited to,
encoding different heterologous proteins, catalytic
30   function, regulatory functions. An exogenous RNA segment
may also be an anti-sense RNA to a nucleic acid segment
that occurs naturally in a target cell, or that may occur
in a target cell if the cell is infected by a virus, and
whose function is intended to be blocked, interrupted or
35   otherwise interfered with by hybridization with the
recombinant STMV genome of the invention which comprises
the exogenous, anti-sense segment.

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The amin acids, which occur in the various
amino acid sequences referred to in the specification
have their usual, three- and one-letter abbreviations,
routinely used in the art, i.e.:

Amino Acid	Abbreviation	
L-Alanine	Ala	A
L-Arginine	Arg	R
L-Asparagine	Asn	N
L-Aspartic acid	Asp	D
L-Cysteine	Cys	C
L-Glutamine	Gln	Q
L-Glutamic Acid	Glu	E
L-Glycine	Gly	G
L-Histidine	His	H
L-Isoleucine	Ile	I
L-Leucine	Leu	L
L-Lysine	Lys	K
L-Methionine	Met	M
L-Phenylalanine	Phe	F
L-Proline	Pro	P
L-Serine	Ser	S
L-Threonine	Thr	T
L-Tryptophan	Trp	W
L-Tyrosine	Tyr	Y
L-Valine	Val	V

The term "transcription vector" refers to
vectors capable of transcribing cDNA sequences contained
therein into the corresponding RNA sequences, where such
sequences are in operational association with other
sequences capable of effecting their transcription, i.e.
promoter sequences for an RNA polymerase. In general,
transcription vectors usually used in recombinant DNA
technology are often in the form of "plasmids", i.e.
circular, double-stranded DNA loops which in their vector
form, are not bound to the chromosome. In the present
specification the terms "vector" and "plasmid" are used

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int rchangeably. However, the inv ntion is intended to
include other f rms of transcription vectors as well,
which function equivalently.

5   The term "a site that is non-essential for RNA
replication" is used to refer to an RNA fragment that is
able to tolerate the presence of a non-viral sequence
without disrupting RNA replication.

            The term "co-infection" (by an STMV-originated
RNA and a helper virus of STMV) and grammatical
10   variations thereof, mean that two independently
replicating virus RNA molecules are caused to occur in a
host cell. The term contains no restriction whatsoever,
concerning the method and order of infection with the
satellite and helper RNA.

15   The transformation of plants cells can be
carried out by any method known in the art for
introducing RNA or cDNA into plant cells, tissues,
protoplasts or whole plants. RNA or cDNA as well as the
helper virus, are preferably introduced into whole plants
20   by mechanical inoculation. Inoculation may be with RNA
alone or with virions containing the desired RNA. The
selection of the best suited transformation protocol
including the manner and parameters of transformation,
timing of transformation, etc. is well within the
25   knowledge of persons of ordinary skill in the art.

            The STMV-based transformation/expression system
has a broad host range, including many economically
important species. The plant hosts include herbaceous
plants such as solanceous plants, e.g. tobacco, tomato,
30   etc.

            The invention is further illustrated by the
following non-limiting examples. The techniques utilized
in carrying out the invention are well known to those of
ordinary skill in the arts of molecular and plant biology
35   and plant virology. The employed methods are fully
described, either directly, or by reference to literature
refer nces disclosing their detailed description. Unless

11. A vector according to Claim 10 selected from
the group consisting of pSTMV+2Δ6, pSTMV+2Δ2, and
pSTMV+2ΔOP.

12. An infectious recombinant RNA molecule derived

5   from STMV ssRNA, having an exogenous RNA segment at a
site that is non-essential for RNA replication in a host
cell.

13. An infectious recombinant RNA molecule
according to Claim 12, wherein said exogenous RNA segment

10   encodes a protein.

14. An infectious recombinant RNA molecule
according to Claim 12, wherein said exogenous RNA segment
comprises RNA having regulatory or catalytic properties.

15. An infectious recombinant RNA molecule

15   according to Claim 13 wherein said exogenous RNA segment
is derived from a plant virus.

16. An infectious recombinant RNA molecule
according to Claim 14 wherein said exogenous RNA segment
is derived from a plant virus.

20   17. An infectious recombinant RNA molecule
according to Claim 13 wherein said exogenous RNA segment
is derived from an animal virus.

18. An infectious recombinant RNA molecule
according to Claim 14 wherein said exogenous RNA segment

25   is derived from an animal virus.

19. An infectious recombinant RNA molecule
according to any one of Claims 12 to 18, wherein said
exogenous RNA segment is located within the region
encoding the coat protein of STMV.

30   20. A DNA transcription vector containing a
substantially full-length cDNA copy of a recombinant RNA
molecule according to any one of Claims 12 to 19.

21. A DNA transcription vector according to Claim
20, selected from the group consisting of

35   pSTMV+2Δ6ACC::CAT (+), pSTMV+2Δ2ACC::CAT, and
pSTMV+2ΔOPACC::CAT.

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oth rwise indicat d, the materials used ar commercially
available, or are in wide circulation among pers ns
practicing in the pertinent arts.

Exogenous gene expression studies were
5   performed with the bacterial chloramphenicol acetyl
transferase (CAT) gene, but other genes the expression of
which produces an easily detectable product, such as the
luciferase gene from firefly, Photinus pyralis, may
equally be used in gene expression studies.
10

Example 1
Cloning of STMV genome
     a.   Source of STMV
           STMV was purified and separated from TMV U5 by
15   rate zonal density gradient centrifugation as described
by Valverde and Dodds, J. Gen. Virology 68, 965 (1987).
RNA was isolated from purified STMV by either the sodium
perchlorate [Wilcockson and Hull, J. Gen. Virology 23,
107 (1974)] or the proteinase K procedure [Maniatis et
20   al., "Molecular Cloning: A Laboratory Manual," Cold
Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
(1982)]. RNAs were denatured in 8 M urea, heated at 60°C
for 3 min., and electrophoresed through 1% low gelling
temperature agarose (Bethesda Research Laboratories).
25   The intact full-length RNA was recovered from the soft
agarose by the freeze/thaw procedure of Benson, BioTech,
March/April, 66-67 (1984).

     b.   Direct enzymatic sequencing of STMV RNA 3' and
30   5' ends and PEI cellulose determination of
           terminal residues
           To determine the complete sequence of the STMV
RNA genome, several strategies were utilized. Use of
these different strategies yielded sequence data which
35   ruled out possible ambiguities encountered in the use of
any single methodology. This was particularly important

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in th determination of the 3'- and 5'-terminal sequences
of the genome.

        The 3'- and 5'-terminal residu s of STMV RNA
were identified by PEI cellulose (Polygram cell 300
PEI/UV$_{254}$, Brinkmann) thin layer chromatography (Buzayan
et al., Virology 151, 186 (1986)).

        Labeling of the 3' terminus of STMV RNA was
with [5'-^{32}P]pCp [Amersham] using T4 RNA ligase [Donis-
Keller et al., Nucleic Acids Res. 4, 2527 (1977)].
Labeling of the 5' terminus of STMV was with [γ^{32}P]ATP
using the forward reaction of T4 polynucleotide kinase
(Maniatis, et al., supra).

        The 5' terminal nucleotide of kinase labeled
STMV RNA was identified by PEI thin layer chromatography.
Intact STMV RNA did not require phosphatase treatment to
achieve efficient labeling with polynucleotide kinase.
Treatment of RNA with TAP and/or BAP prior to using
kinase did not increase the efficiency of labeling the 5'
termini of STMV RNA. The nucleotide detected after
nuclease P1 digestion of treated RNA was predominantly
adenosine, indicating that the majority of the 5' termini
of STMV RNA encapsidated in virions have a non-
phosphorylated adenosine residue.

        The 3' terminal nucleotide of T4 RNA ligase-
labeled STMV RNA was identified by PEI thin-layer
chromatography. The only nucleotide detected after RNase
T2 digestion was adenosine.

        Direct enzymatic (Donis-Keller et al., supra)
sequencing of the 3' end of STMV RNA was conducted as
previously described (Zuidema et al., Proc. Natl. Acad.
Sci. (USA) 83, 5019 (1986)). These sequence data were
used to construct an oligonucleotide for use in the
production of a cDNA library.

        c.   Method of cloning
        First strand cDNA synthesis from STMV ssRNA was
primed using sonicated and DNas -treated calf thymus DNA

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(Maniatis et al., Supra), or by using a
d oxyoligonucleotide c mplementary to the 3' 16 terminal
nucleotid s of STMV RNA. The s quence of this
deoxyoligonucleotide was based on RNase determined

5   sequence data. First strand cDNA synthesis was also
primed using oligo dT (12-18) (Maniatis et al., supra) on
STMV RNA that had been polyadenylated at its 3' terminus
as described by Gething et al., Nature 287,301 (1980).
        Second strand synthesis was performed using

10   RNase H and DNA polymerase I (Bethesda Research
Laboratories), essentially as described by Gubler and
Hoffman, Gene 25, 263 (1983). The resulting double
stranded cDNA was either digested with TaqI and ligated
into the AccI site of pUC118 or pUC119 plasmid (Vieira

15   and Messing, Production of Single-Stranded Plasmid DNA,
In "Methods in Enzymology" (R. Wu and L. Grossman, Eds.
153, 3-11 Academic Press, New York (1987)), or was made
blunt-ended with T4 DNA polymerase as described by Gubler
and Hoffman, supra, and ligated into the SmaI site of

20   plasmid pUC118 or pUC119. Ligation products were used to
transform E. coli strain DH5α (Bethesda Research
Laboratories).
        Ampicillin resistant transformants were
selected by colony hybridization with kinase-labeled STMV

25   virion RNA probe (Rezaian et al., Virology 131 221
(1983)). Nick-translated DNA from four of the largest
plasmids averaging 450 base pairs hybridized only to STMV
RNA in Northern hybridizations, which confirmed insert
specificity.

30

        d.   Sequencing of cDNA clones to determine the
             sequence of the STMV genome
             Recombinant plasmids were purified from 29
ampicillin-resistant colonies that hybridized with

35   kinase-labeled STMV RNA probe. Their inserts were
excised by digestion with TaqI, or KpnI and PstI and
resolved by electrophor sis in agaros gels. Sequencing

was by the dideoxynucleotide chain termination method (Sanger et al., Proc. Natl. Acad. Sci. (USA) 74 5463 (1977)). Certain of the inserts wer sequenced in both directions. When ambiguities arose and there was a need

5   to confirm sequence data, genomic RNA itself was sequenced using deoxyoligonucleotides as primers. Thus, from the 3'-terminus to about ribonucleotide 830 and from about nucleotide 470 to the 5'-terminus of the genomic RNA were sequenced directly. The 29 cDNA fragments,

10   including those in clones pSTMV-2, pSTMV-13, and pSTMV-31 (Fig. 3), represented the entire genome of STMV with the exception of the nine 5'-terminal nucleotides.

          To complete the 5' sequence data of STMV RNA, a deoxyoligonucleotide (5'-GGCGACTGAAGGCC) complementary to

15   nucleotides 102-115 was used to prime reverse transcription in the presence of dideoxynucleoside triphosphates (Palmenberg et al., Nucl. Acids Res. 12, 2969 (1984)). The sequence of the 5' terminus of STMV RNA thus was deduced from gels in the standard fashion.

20   RNase determined sequence was used for confirmation. RNase determined sequence was also used to confirm that cDNA clones covered the the 3' terminus.

          The primary structure of STMV RNA, shown in Fig. 1, is 1,059 nucleotides long. Restriction maps of

25   six of the larger cDNAs used to deteremine the sequence in Fig. 1 were obtained using 30 different restriction enzymes; these maps conformed exactly to the maps predicted from the nucleotide sequence in Fig. 1.

30   Example 2
         a.   Sequencing of clone inserts
          The STMV RNA sequence was screened for potential coding regions in all three reading frames in both the virion (positive) and complementary (negative)

35   senses. Two open reading frames (ORFs) with the potential to code for proteins of 6,800 Mr and 17,500 Mr were observed. The first ORF (Fig. 1) begins at the

first 5' AUG triplet in positions 53 to 55, and ends with
a UGA termination codon at bases 227 to 229. The largest
ORF (Fig. 1) b gins at the second AUG triplet in
positions 163 to 165 and ends with a UGA termination
5   codon at residues 640 to 642. The 418 nucleotides
following the ORF for the capsid protein do not encode
any polypeptides longer than 16 amino acids. The
negative sense RNA has a single ORF which had the coding
capacity for an 8K protein.
10   The 12 N-terminal amino acids of the capsid
protein, corresponding to the first 12 codons of ORF 2,
are underlined in Fig. 1. There is a perfect fit between
the predicted amino acids and those determined by amino
acid sequencing from the N-terminus. The results of an
15   amino acid composition analysis agreed well with the
empirically determined amino acid composition of the STMV
capsid protein indicated in Fig. 1.

       b.   Characterization of sequence
20   One microgram of STMV RNA was used in each
translation reaction in either a rabbit reticulocyte
lysate or a wheat germ extract cell-free system (Promega
Biotec, Madison, WI, U.S.A.) using ^{35}S labeled methionine.
Time course analysis of cell-free translation products
25   was achieved by removing one microliter aliquots from a
single reaction mixture at 1 min. intervals. ^{35}S labeled
polypeptides were denatured by boiling in 63 mM Tris-Cl,
pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol
(denaturing buffer), then electrophoresed through 15%
30   polyacrylamide, 0.1% SDS gels (Laemmli, Nature 227, 680,
(1970)). Gels were soaked in three changes of 30%
methanol/10% acetic acid, vacuum dried at 60°C, and
exposed to Kodak X-Omat AR film for 24 to 72 hr. STMV
RNA efficiently directed the synthesis of two
35   polypeptides in both wheat germ and rabbit reticulocyte
systems.

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Samples for Western-blot analysis included
cell-free translation products, STMV viri ns, TMV U5
virions, homogenized tobacc tissue infected with
STMV/TMV U5, and homogenized non-infected tobacco tissue.

5   Samples were treated and electrophoresed as described
above. After electrophoresis, proteins were
electrophoretically transferred to a nitrocellulose
membrane (Trans-Blot Cell, Bio-Rad). After
immobilization of the proteins to the membrane, the blot

10   was developed as described by Blake _et al._, _Anal._
Biochem. 135, 175 (1984) using a 1/1000 dilution of an
STMV specific antiserum (Valverde and Dodds, _supra_).
Western-blot analysis of these _in vitro_
translation products revealed that the larger protein had

15   the same electrophoretic mobility as the STMV capsid
protein (17,500 Mr) and an equivalent protein found in
sap from STMV infected plants. These proteins reacted
with an antiserum specific for STMV. The second protein
(6,800 Mr) and TMV capsid protein did not react with STMV

20   antiserum. The 6,800 Mr protein corresponds in size to
the putative protein coded by the first ORF in the
genome. Time course analysis of _in vitro_ translation
demonstrated that the ratio between the amounts of the
6,800 Mr protein and the 17,500 Mr protein were similar

25   at each sampling time. No significant amount of higher
molecular weight proteins were synthesized at any
sampling time.
It can be concluded from size analysis of cell-
free translation products, serological analysis, amino

30   acid sequence and composition results that ORF 2 encodes
the 17,500 Mr STMV capsid protein.

Example 3
        a.   Construction of pSTMV+2Δ6

35   Methods for the synthesis of cDNA clones were
as herein above described. Freshly prepared STMV ssRNA
purified from STMV virions was obtained from J. A. Dodds,

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University of California, Riverside. Cl nes pSTMV-2,
pSTMV-13, and pSTMV-31 were constructed at the University
of California, Riverside, following Example 1 and
obtained from J. A. Dodds. As shown in Figure 3, 100
5   nanograms of insert DNA fragments from these clones were
ligated at a unique _DraI_ and unique _BglII_ site. This
ligation mixture was transformed into E. coli strain
DH5α, and transformants were selected on ampicillin
plates. Plasmid DNA isolated from these transformants
10   was digested with _KpnI_ and _HindIII_ to identify near full-
length STMV cDNA clones. One of these clones, pSTMV-3,
which contains a 986 base pair insert which lacks the 73
5' terminal nucleotides of STMV, was sequenced to confirm
identity with the STMV consensus sequence and then used
15   for the construction of a full-length STMV cDNA clone.
          To generate 5'-terminal clones, an
oligonucleotide complementary to nucleotides 351-362
(5'-GGAATCTGTCCGG) of the STMV genomic RNA was used to
prime first-strand cDNA synthesis. A second primer
20   (5'-GGTACCAGTAAAACTTACCAATCAAAA) complementary to the 3'
end of the first-strand cDNA was then used to
specifically prime second strand cDNA synthesis of only
those molecules that extend through (are complementary
to) the final 5'-terminal nucleotides of the STMV genome.
25   Included in this oligonucleotide primer was a 6-base
overhang that is the recognition sequence for _KpnI_. This
unique restriction site was used to facilitate cloning of
the 5'-end of the STMV genome without losing 5'-terminal
sequences, which is a deficiency of many alternative
30   cloning strategies.
          Approximately 5 μg of double-stranded cDNA
derived from the above-described reactions was digested
with _KpnI_ and _MstII_ to yield a 249 base pair fragment and
a 113 base pair fragment. These fragments were not
35   separated as only the 249 base pair fragment contained
both the _KpnI_ 3' overhang and _MstII_ 5' overhang. pSTMV-3
DNA was dig sted with _KpnI_ and _MstII_; the resulting

vector fragment was gel purified, and ~50 nanograms was
ligated with ~ 100 nan grams f the 249 base pair KpnI-
MstII cDNA fragment. This ligation mixture was used to
transform E. coli strain DH5α, and transformants were

5   selected on ampicillin plates. Transformants containing
5' terminal sequences were identified by colony
hybridization with an oligonucleotide
5'-GCATTAATGACCACGACAGTCCTGGTGGTTAGGTCTTTTGATTGGTAAGTTT
TATTGGTACC) complementary to the 5' end of genomic STMV

10   RNA. Clones thus identified were anticipated to contain
full-length copies of the STMV genome.

       Sequence analysis (Sanger dideoxynucleotide
method on denatured plasmid DNA using ^{35}S-dATP) indicated
that all nine clones derived from these experiments fell

15   short of the 5' terminus by nine nucleotides. The
reasons for this were unclear, although sequence analysis
indicated that there was a tetranucleotide sequence
within the STMV genome, i.e., bases nine to twelve
(5'-TTAC), at which KpnI (recognition sequence 5'-GGTACC)

20   may have digested the double-stranded cDNAs. New England
Biolabs has recently reported apparent star activity in
preparations of KpnI, supporting the hypothesis that KpnI
could cut these clones at this site. One of these
clones, pSTMV-KMB35, was used in further constructions

25   designed to yield a full-length STMV clone.

       To produce insert DNAs containing the missing
nine 5'-terminal nucleotides, an alternative cloning
strategy was used. First-strand cDNA synthesis was
primed using an oligonucleotide (5'-CCCTTCGATTTAAG)

30   complementary to nucleotides 940 through 958. This was
chosen such that a unique restriction enzyme site (BglII)
could be utilized for forced orientation cloning using
pSTMV-KMB35 as a vector, as sequences 3' of this site are
difficult to clone due to secondary structure. Second

35   strand synthesis was conducted using an oligonucleotide
to prime second strand synthesis of only molecules that
extend to the 5' terminus. This oligonucleotide

(5'-CTGCAGATCTAGAATAAAACTTACCAATCAAAAG) utilized a unique
XbaI ext nsion to vercome the problem discussed above
which result d in clones that were missing th nine 5'-
terminal nucleotides. This restriction site (XbaI) also
5   provided the basis for a convenient method for subcloning
insert DNA into the pMJ5 transcription vector (described
in detail in Janda et al., Virology 158, 259 (1987)) in
such a way that only two non-viral nucleotides are
included in transcripts derived from this construct.
10   Approximately 5 µg of this double-stranded cDNA
was digested with XbaI and BglII to yield a 791 and a 149
base pair fragment. The 791 base pair fragment was not
further purified because only it contained both the XbaI
5' overhang and the BglII 5' overhang. pSTMV-KMB35 DNA
15   was digested with XbaI and BglII and gel purified..
Approximately 100 nanograms of the 791 base pair XbaI-
BglII cDNA fragment was ligated with ~ 50 nanograms of the
vector fragment. This ligation mixture was transformed
into E. coli strain DH5α, and transformants were selected
20   on ampicillin plates. Plasmid DNA was prepared from a
transformant (pXBH118A); when digested with XbaI and
HindIII, an insert fragment of ~ 1060 base pairs was
produced. This insert DNA was sequenced by the Sanger
dideoxynucleotide method on denatured plasmid DNA using
25   ^{35}S-dATP, and the sequence data revealed that the missing
nine 5'-terminal nucleotides had been cloned in clone
pXBH118A. However, three transitions had occurred in the
3' non-coding region of the genome: C to U at position
682, A to C at position 751, and C to U at position 753.
30   Insert DNA derived form this clone (pXBH118A) was
utilized in construction of an RNA transcription vector
pSTMV+2Δ6 as described below.
Five µg of pMJ5 DNA was digested with StuI and
HindIII, and the resultant vector DNA fragment was gel
35   purified. Insert DNA was prepared from pXBH118A DNA by
digestion with XbaI followed by tr atment with mung bean

25

nuclease to remov the non-viral nucleotides. This DNA
preparation was then digested with HindIII. After gel
purification, ~ 100 nanograms of the resultant 1059 base
pair fragment was ligated with 100 nanograms of the
5   prepared pMJ5 vector DNA. This ligation mixture was
transformed into E. coli strain DH5α, and transformants
were selected on ampicillin plates. Plasmid DNA purified
from these transformants was digested with KpnI and
HindIII to identify those containing an insert fragment
10   of ~ 1120 base pairs. The insert DNA from one clone,
pSTMV+2Δ6, was sequenced by the Sanger dideoxynucleotide
method on denatured plasmid DNA using ^{35}S-dATP to confirm
that this clone contained the correct sequence. It was
determined from the sequence data that the insert DNA
15   contained three errors in addition to the three
transitions that are described above. To reflect the
presence of these six errors, this vector was designated
pSTMV+2Δ6. To correct all six errors, another vector,
pSTMV+2Δ1, otherwise referred to herein also as
20   pSTMV+2Δ2, was constructed.

     b.   Construction of pSTMV+2Δ1
     Approximately five μg of pSTMV+2Δ6 DNA was digested
with SalI and HindIII, and the fragments were separated
25   by agarose gel electrophoresis. The ~ 4300 base pair
fragment comprising the vector and 5' end of the STMV
genome was purified.
     An oligonucleotide
(5'-GATTTAAAGCTTGGGCCGCTTACCCGCGGTTAGGG-3') complementary
30   to the correct 3' terminal sequence of the STMV genome
and containing a unique HindIII extension was
synthesized. This oligonucleotide was used to prime
first-strand synthesis of cDNA. The resultant cDNA
molecules were made double-stranded, digested with SalI
35   and HindIII, and the 450 base pair SalI-HindIII fragment
was purified from an agarose g l. One hundred nanograms

of this fragment was ligated with fifty nanograms of the
HindIII-SalI fragm nt derived from pSTMV+2Δ6. This
ligation mixture was transform d into E. coli strain
DH5α, and transformants and digested with KpnI and

5   HindIII to identify clones containing an ~ 1120 base pair
insert fragment. The insert DNA was sequenced by the
Sanger dideoxynucleotide method on denatured plasmid DNA
using ^{35}S-dATP. This sequence analysis indicated that all
six errors present in pSTMV+2Δ6 had been corrected.

10   However, two new errors (a C to G transition at
nucleotide 1057 and a C to T transition at nucleotide
984) had been introduced, and the resultant vector was
first designated pSTMV+2Δ1, later changed to pSTMV+2Δ2,
to reflect these two nucleotide differences from the STMV

15   RNA genome.

        c. Construction of pSTMV+2ΔOP
              A 3' PstI site was introduced at the 3' end of
        the STMV sequence through use of the oligonucleotide:
20   5'-ATCTGCAGGGCCGCTTACCCGCGGTTAGGG-3'. This
        oligonucleotide is complementary to the 3' terminal 24
        STMV nt sequence and additionally contains a PstI
        recognition site. The oligo was used to prime first
        strand cDNA synthesis from STMV RNA. Second-strand was
25   synthesized by the nick-translation method using RNAse H
        and DNA polymerase I (Gubler and Hoffman, 1983). The
        resultant double-stranded cDNAs were madeblunt-ended with
        T4 DNA polymerase and digested with PstI. 50 ng of
        insert were ligated to 100 ng of SmaI-PstI-digested
30   pUC118 and the ligation was transformed into DH5-alpha
        cells and amp[R] colonies were selected. Correct plasmid
        was first identified by bands which resulted from
        digestion with PstI and BglII, and then confirmed by DNA
        sequencing. One plasmid that contained the PstI site and
35   the correct 3' terminal 500 nt was called
        pSTMVPstOligo#6.

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Plasmid pSTMVPstOligo#6 was digested with <u>Eco</u>RI and
<u>Bgl</u>II and the ~3390bp fragment was isolated on a 1.0%
agarose gel. 50 ng of this fragment were ligated to 100
ng of the 833 bp <u>Eco</u>RI-BglII fragment of pSTMV+2Δ2
5   isolated from a 1% agarose gel. The ligation was
transformed into DH5-alpha cells and amp^R colonies were
selected. Correct plasmid was identified by sequencing,
and was called pSTMV+2ΔOP. The insert in this plasmid
has the exact sequence of STMV, as determined in
10   Example 1.

       d.   Construction of pSTMV+2ΔHPA

            Plasmid pSTMV+2Δ2 was digested with <u>Hpa</u>I and
the large vector fragment was isolated on a 1.0% agarose
15   gel. The ends of the isolated vector fragment were
religated and the ligation mixture was transformed into
DH5-alpha cells. Amp^R colonies were selected. Correct
plasmid exhibited one band of 3850 bp upon digestion with
<u>Hpa</u>I, and was called pSTMV+2ΔHPA. This plasmid contains
20   a deletion from nucleotides 414 through 787 of the STMV
coat protein and 3' noncoding sequence.

       e.   Analysis of RNA transcripts

            pSTMV+2Δ6, pSTMV+2Δ2, pSTMV+2ΔOP, and
25   pSTMV+2ΔHPA were used to produce transcripts that have
only two non-viral nucleotides at the 5' terminus, in
adition to six, two or zero transitions at the 3'-end, or
a deleteion at the 3'-end, respectively. Forty μg of
vector DNA were linearized with <u>Hind</u>III (2Δ6, 2Δ2,
30   2ΔHPA), and then treated with 20 units of mung bean
nuclease (New England Biolabs) to remove the four non-
viral nucleotides contained in the <u>Hind</u>III 5' overhang,
or linearized with <u>Pst</u>I (2Δ6), and then, in all four
cases, phenol\chloroform extracted, and ethanol
35   precipitated. Ten μg of these DNAs were incubated in the
presence of 100 units of T7 RNA polymerase in a total
volume of 100 μl containing 40 mM Tris-HCl, pH 7.5, 6 mM

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MgCl$_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 40 units
RNasin, and 2.5 mM each ATP, CTP, UTP, and GTP.
Reactions were incubated at 37°C for two hours and
typically yielded 60-70 μg of transcribed RNA. The
5   transcripts resulting from pSTMV+2Δ6 and pSTMV+2Δ2
migrated with the same mobility as did wild type STMV RNA
on denaturing agarose gels. The transcripts from
STMV+2ΔOP similarly migrated with the same mobility,
while those from pSTMV+2ΔHPA were smaller than wild-type
10   STMV.

      f.   In vitro translation
            Transcripts derived from pSTMV+2Δ6 and
pSTMV+2Δ2 translated efficiently in a wheat germ cell-
15   free extract translation system (Promega Biotec) and
produced polypeptides identical in size and relative
amounts to those produced from wild type STMV ssRNA.

      Example 4
20   Infectivity studies
      a.   Infectivity protocol
            For infectivity studies, 0.9 ml of a mixture
containing 0.05 M glycine, 0.03 M K$_2$HPO$_4$, pH 9.2, 1%
sodium pyrophosphate, 1% Macaloid, and 1% Celite,
25   adjusted to pH 8.5 with H$_3$PO$_4$, was added to a 100 μl
transcription reaction mixture. One hundred μl of this
1000 μl mixture was utilized for each mechanically
inoculated tobacco (N. tabacum cv. Xanthi) leaf.
Inoculations were on leaves systemically infected with
30   the TMV U5 or U2 helper virus. Alternatively, leaves
were inoculated with helper virus 0, 1, 2 or 6 days
before inoculation with transcript RNA.
            A mixture of two different tobamoviruses were
used in this study, TMV U2 and TMV U5. TMV U5 is the
35   natural helper virus for STMV. TMV U5 was obtained form
J.A. Dodds, University of California, Riverside, and was
deriv d from a stock of Nicotiana Tabacum cv. Xanthi

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originally develop d and maintained by L.G. Wheath rs.
TMV U2 is available from the American Type Culture
Collection, Rockville, Mareyland, USA and is essentially
the same as TMV U5, merely having been isolated first at

5   a geographical location different from that at which TMV
U5 was first isolated. The isolates used had been
passaged through single local lesions in _Nicotiana
tabacum_ cv Xanthi nc. prior to inoculation to _N.tabacum_
cv Xanthi, which was the host used for maintaining

10   isolates. Stock plants harboring the viruses have been
tested repeatedly for the presence of STMV and have
always tested negative. However, prior to each use as a
source of inoculum, extracts from the maintenance hosts
were tested for STMV contamination by dot spot

15   hybridization or immunodiffusion assay. All plants
indexed negatively throughout the experimental period.

TMV U5-infected _Nicotiana tabacum_ cv. Xanthi
are being maintained as stock plants under standard
conditions (Valverde and Dodds, _Supra_ and _J. Gen._

20   _Virology 67_, 1875 (1986)). One of the two TMV U5
isolates used was determined by dsRNA analysis (detection
of a pair of RF dsRNAs with slightly different mobilities
and other pairs of dsRNAs with strain-specific
mobilities) and host reaction in _N. sylvestris_ (local

25   lesion formation indicative of TMV U1) to be contaminated
by readily detectable levels of TMV U1 and is referred to
as TMV U5B to distinguish it from TMV U5A which was not
delectably contaminated with TMV U1 by these criteria.

Freshly prepared STMV ssRNA, purified from STMV

30   virions, was obtained from J.A. Dodds, University of
California, Riverside.

Seeds of _N. tabacum_ cv. Xanthi were provided by
J.A. Dodds, University of California, Riverside. Plants
were grown and maintained under standard conditions.

35

b. STMV Assays
1. Immunoanalysis for STMV coat protein

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T n to 21 days post-inoculation, the uppermost
2-3 non-inoculated systemically infected leaves (1-2 g
fresh weight) were harvested for analysis. Ouchterlony
double-immunodiffusion assays were carried out to screen
5   for the presence of STMV coat protein antigen and to look
for serological differences between a wild type STMV
isolate in a mixed infection with TMV U5, and progeny
virions from RNA transcript infections. One g of leaf
tissue was ground in 1 ml of 0.14M NaCl, the extract was
10   filtered through Miracloth and 50 μl of the sample was
placed in alternating wells (wild type sample next to
experimental sample) cut in a 1.0% agarose, 0.1M NaCl
plate. An STMV specific antiserum was used in the center
well (Valverde and Dodds, 1987, supra). Initial results
15   were recorded after 24 hr. incubation at room
temperature; examination for spur formation between
adjacent samples was stopped six days later.

2.   Nucleic acid analysis of STMV sequence
20   Total nucleic acids were extracted as described
by Dunsmuir et al., and spotted onto Zeta-Probe membrane
(BioRad). Alternatively, 20 μl of each test sample used
for the Oucterlony assay were placed in a microfuge tube
and centrifuged for 6 minutes. Two to five μl were
25   applied to a nitrocellulose membrane, air dried and baked
at 80°C for 2 hours. Blots were assayed with
^{32}P-labelled plasmid pSTMV2, which contains a cDNA insert
that represented nucleoctides 70 to 405 of the STMV
genome, or with RNA transcript from similar plasmids, or
30   cDNA transcribed from STMV genomic RNA. Extracts from
non-inoculated plants or plants infected with any helper
virus alone did not give a positive signal with any of
the probes.

35   3.   Analysis of STMV virions
Wild-type STMV or transcript-derived virions
were purified and s parated from TMV U5 and U2 by rate

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zonal density gradient centrifugation as described by
Valverde and Dodds (1987). Relative banding positions
were compared between wild-type and transcript-derived
virions. Samples of gradient-purified virions were
5   negatively stained with 2% uranyl acetate and examined by
electron microscopy. RNA was isolated as described in
Example 1.a, supra.

     c.   Analysis of results of infectivity studies
10   1. Preliminary study with pSTMV+2Δ6 and
pSTMV+2Δ2

          At 15 days or 21 days post-inoculation, leaf
tissue was harvested from infected plants, and total
nucleic acid was prepared according to the first three
15   steps of the procedure described in Dunsmuir et al.,
Plant Molecular Biology Manual C1: 1-17 (1988). These
nucleic acids were then applied to nitrocellulose filters
for dot blot hybridization assays using nick-translated
pSTMV2 as a probe. Transcripts derived from pSTMV+2Δ6
20   yielded three infected plants out of a total of eight
plants inoculated with transcripts. Transcripts derived
from pSTMV+2Δ2 yielded 100% infectivity.

          To rule out the possibility that a wild type
STMV contaminant was responsible for the production of
25   virus in plants inoculated with in vitro transcripts,
virions were purified from plants which had been
inoculated with transcripts derived from pSTMV+2Δ6. RNA
was purified from these virions, and sequence data were
obtained. These data included a region of the genome in
30   which pSTMV+2Δ6 differs at three positions from the wild
type. As expected, the C to T transition at nucleotide
position 681, the A to C transition at nucleotide
position 751, and the C to T transition at nucleotide
position 753 all were retained in the virion RNA prepared
35   form plants inoculated with in vitro transcripts. These
results indicated the in vitro transcripts, and not a
wild type contaminant, were responsible for the

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pr duction of virus in these plants. This result also
implied that at least some changes in nucleotide sequence
could be accommodated by the virus without loss of
infectivity. Whether such changes could include
5   additional sequences and/or deletions was explored in the
development of recombinant STMV vectors.

        2. Four further experiments
            a. Infectivity
10   Data were obtained from several subsequent
experiments involving coinfection of plants with
pSTMV+2Δ2 and TMV U2 as helper followed by analysis of
leaves 10 days after inoculation. STMV in vitro
transcripts that had been stored frozen for up to 12
15   weeks retained high levels of infectivity. 100%
infection was obtained with concentrations from 5 to 50
μg/ml. Incidence of infection by STMV dropped to 75% at
0.5 μg/ml and was not detected when 0.1 μg/ml was used.
            Further experiments were carried out with
20   analysis of leaf tissue between 10 and 21 days post-
inoculation with STMV transcript at 50 μg/ml, used either
either without storage or after storage for four weeks.
It was possible to obtain 100% infectivity of plants by
STMV in most experiments when TMV-U5-A or TMV U2 were
25   used as helper viruses and when pSTMV+2Δ2 and pSTMV+2ΔOP
were used to produce transcripts. Delay of 0 hours or 48
hours between inoculation with TMV and inoculation with
STMV transcript appeared to have no effect on infec-
tivity. When TMV U5-B was used with these transcripts,
30   the incidence of infection ranged from 25% to 100%
depending on the experiment. The lowest incidences of
infection (25-50%) were obtained when TMV U5B was the
helper and pSTMV+2Δ6 was used to produce transcripts; the
percentages were near 100 % with pSTMV+2ΔOP and
35   pSTMV+2Δ2.

            cDNA constructs also were prepared to produce
(-) sens in vitro transcripts. Thes transcripts were

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not infectious when inoculated under the conditions
described abov .

  When large plants were pre-infected for 48
hours with TMV before inoculation with _in vitro_
5 transcripts from pSTMV+2Δ2, it was possible to approach
100% infectivity with the STMV construct. The same
result was achieved with small plants. No difference in
the incidence of infection was seen when helper virus and
STMV RNA transcripts were inoculated together or when
10 there was a 24 or 48 hour delay after TMV inoculation
before inoculation with the transcripts. Shading plants
prior to inoculation provided no advantage, nor did it
reduce the incidence of infection.

  b. Analysis of progeny virions and RNAs
15   Virions indistinguishable from STMV accumulated
in plants infected with _in vitro_ transcripts from
pSTMV+2ΔOP, pSTMV+2Δ2, and pSTMV+2Δ6, as determined by
electron microscopy of negatively-stained particles,
analytical sucrose density gradient centrifugation, and
20 Ouchterlony immunodiffusion assay. Similar amounts (4.6
to 4.8 mg/10 g fresh leaf tissue) of STMV virions were
purified from plants infected with either STMV or with
the three different transcripts. Two major translation
products (17.5 Kd and 6.8 Kd) were detected when STMV
25 RNA, transcripts from pSTMV+2Δ2, and progeny RNA from
virions derived from pSTMV+2Δ2 transcript infections were
used to direct synthesis of peptides in a wheat germ
cell-free translation system.

  Primer extension in the presence of
30 dideoxynucleotides and RNase sequence analysis were
performed to determine if any of the eight different
single nucleotide transitions were maintained in the
progeny from plants inoculated with STMV _in vitro_
transcripts. RNAse sequence analysis of progeny RNA
35 derived by infection with _in vitro_ transcripts prepared
from pSTMV+2Δ2 showed that transitions at positions 989
and 1057 were not maintained and that the 3'terminal

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sequ nce of progeny was id ntical to that of wild-type.
RNAs sequence analysis of progeny derived infection with
in vitro transcripts prepared from pSTMV+2∆6 gave a
similar result in that the transitions introduced at
5   positions 1053 and 1046, and the insertion at position
1055 had reverted to wild-type sequence. However, a
single deletion not present in the transcript was
detected at residue 1050 causing the sequence to differ
from wild-type. Primer extension analysis of this
10   progeny RNA in the region from positions 682 to 753
showed that the three transitions in this region were
retained.

        The 5' termini of STMV, _in vitro_ transcripts,
and progeny derived by infection with these transcripts
15   were examined to determine if the non-viral Gs
(guanylates) present in the transcripts were retained in
the progeny. Primer extension in the presence of
dideoxynucleotides indicated that the progeny RNA was the
same length as wild-type STMV RNA and that the
20   transcripts were at least two nucleotides longer than
wild-type RNA.

        Example 5
        Construction of recombinant STMV vectors
25   a.   Recombinant STMV-CAT vector system
                 1. pSTMV+2∆6/CAT
                 The bacterial chloramphenicol acetyl
transferase (CAT) gene was inserted into vector pSTMV+2∆6
near the 3' end of the coat protein coding region into
30   the AccI site at nucleotide position 604. The CAT gene
was excised from plasmid pCaMVCN (Pharmacia) as a 788
base pair TaqI fragment and gel purified. One hundred
nanograms of this fragment was ligated to 50 nanograms of
AccI-digested pSTMV+2∆6 DNA that had been treated with
35   calf alkaline intestinal phosphatase. The ligation
mixture was transformed into DH5α bacterial cells, and
ampicillin resistant colonies were selected. Plasmids

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containing the CAT gen in the same orientati n as the
viral coat prot in gene yielded two fragments (4155 and
892 bas pairs) upon digestion with EcoRI. The junction
of these two genes results in an out-of-frame fusion
protein; hence, the CAT gene is expressed as a result of
plant ribosomes initiating protein synthesis from the
bacterial CAT initiation codon. This construct was
designated pSTMV+2Δ6ACC::CAT(+).

> 2. Construction of pSTMV+2Δ2/CAT
>   expression vector
>   (pSTMV+2Δ2ACC::CAT(+))

        The CAT gene was inserted into the STMV
sequence near the 3' end of the coat protein-encoding
region in vector pSTMV+2Δ2.

        Plasmid pCaMVCN was digested with TaqI and the
788 bp CAT-encoding fragment was isolated on a 1.0%
agarose gel. One hundred nanograms of the 788 bp
fragment were ligated to 50 ng of AccI-digested, calf
intestinal phosphatase-treated pSTMV+2Δ2. The ligation
was transformed into DH5-alpha cells and amp^R colonies
were selected. Correct plasmid yielded fragments of 850
and 4159 bp upon digestion with EcoRI, and was called
pSTMV+2Δ2ACC::CAT(+). The CAT gene is out of frame in
relation to the STMV coat protein coding sequence.

> 3. Construction of pSTMV+2ΔOP/CAT
>   expression vector
>   (pSTMV+2ΔOPACC::CAT(+))

        Plasmid pSTMV+2Δ2ACC::CAT(+) was digested with
KpnI and BglII and the 3385 bp fragment was isolated on a
1% agarose gel. 100 ng of the 1620 bp KpnI-BglII
fragment from 2Δ2ACC::CAT(+) were ligated to 50 ng of the
3385 bp fragment. The ligation mixture was transformed
into DH5-alpha cells, and amp^R colonies were selected.
Correct plasmid demonstrated bands of 850 and 4159 bp
upon digestion with EcoRI, and was called
pSTMV+2ΔOPACC:CAT(+). The CAT gene is in the proper

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orientation, but is out of frame in relation to the STMV
coat protein coding sequence in this plasmid.

                    4. Infectivity studies

                    In vitro transcripts were prepared from
5   pSTMV+2Δ6ACC::CAT(+), pSTMV+2Δ2ACC::CAT(+), and
        pSTMV+2ΔOPACC::CAT(+) as described previously and used to
        inoculate tobacco plants.

                    Analysis of infected and control plants three
        and six days post inoculation showed that CAT was
10   expressed in the plants infected with
        pSTMV+2Δ6ACC::CAT(+) and was not expressed in plants
        infected with pSTMV+2Δ6.   CAT assays were conducted
        according to Herrera-Estrella et al., Plant Molecular
        Biology Manual B1, S.B. Gelvin and R.A. Shilperoort,
15   Eds., Kluver Academic Publishers, Dordrecht, The
        Netherlands, pp 1-22 (1988).

                    CAT protein was not detected in plants infected
        with pSTMV+2Δ2ACC::CAT(+) and helper U5, however dot/spot
        analysis performed 21 days post inoculation revealed the
20   presence of STMV and CAT nucleic acid in the leaves of
        one of four infected plants.   Thus, pSTMV+2Δ2ACC::CAT(+)
        is weakly infective in combination with helper U5.

                    One-hundred percent of the leaves and systemic
        tissue analyzed from plants infected with
25   pSTMV+2ΔOPACC::CAT and U2 helper revealed the presence of
        CAT and STMV nucleic acid.   Because the CAT gene is out
        of frame in this construct, CAT protein was not expected
        necessarily to be expressed and was not detected.

30   While the present invention has been described
        with some particularity herein, those of skill in the
        pertinent arts will recognize many variations and
        modifications of what has been described that are within
        the spirit of the invention.   It is intended that such
35   variations and modifications are also encompassed by the
        description and following claims.

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CLAIMS:

1. An infectious recombinant RNA molecule derived from STMV ssRNA.

2. An infectious recombinant RNA molecule according to Claim 1 unaccompanied by endogenous components not essential for its infectious properties.

3. An infectious recombinant RNA molecule according to Claim 1, which is made by _in vitro_ transcription of a cDNA.

4. An infectious recombinant RNA molecule according to Claim 3, which is derived from an STMV ssRNA having a nucleotide sequence as shown in Figure 1 or a degenerate equivalent thereof.

5. An infectious recombinant RNA molecule according to Claim 4, which is derived from an STMV ssRNA having the nucleotide sequence as shown in Figure 1.

6. A DNA transcription vector which comprises a substantially full-length cDNA copy of an infectious STMV ssRNA and from which said cDNA is capable of being transcribed.

7. A DNA transcription vector according to Claim 6 wherein the transcript of said substantially full-length cDNA copy of an STMV ssRNA has a nucleotide sequence as shown in Figure 1 or a degenerate equivalent thereof.

8. A DNA transcription vector according to Claim 7 wherein the transcript of said substantially full-length cDNA copy of an STMV ssRNA has the nucleotide sequence shown in Figure 1.

9. A DNA transcription vector according to any one of Claims 6 to 8 wherein transcription of said cDNA is under he control of a promoter for a DNA-dependent RNA polymerase.

10. A DNA transcription vector according to Claim 9, which is a derivative of the plasmid pMJ5 wherein said cDNA is ligated between the _StuI_ and _HindIII_ sites of plasmid pMJ5s.

22. A method of transforming a plant cell, comprising introducing into the cytoplasm of said cell an inf ctious recombinant RNA molecule according to any one of Claims 13 or 14 and a helper virus of STMV.

23. A method according to Claim 22 wherein said helper virus is a strain of TMV selected from the group consisting of U1, U2 and U5.

24. A method according to Claim 22 wherein said RNA molecule and said helper virus are introduced into the cytoplasm of said cell by mechanical inoculation.

25. A method of Claim 22, wherein said cell is that of a herbaceous plant.

26. A method of Claim 25 wherein said cell is that of a solanceous plant.

27. A method of transforming a plant cell, comprising introducing into the cytoplasm of said cell an infectious, substantially full-length cDNA copy of an STMV ssRNA.

28. A method for the production of an exogenous protein in the cytoplasm of a plant cell, comprising co-infecting said plant cell with an infectious recombinant RNA molecule according to Claim 13 or with a cDNA having one strand substantially complementary to said RNA and capable of providing said recombinant RNA by transcription in vivo, and a helper virus of STMV.

29. A method according to Claim 28, wherein said helper virus is a TMV of a strain selected from the group consisting of U1, U2 and U5.

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   1   AGUAAAACUUACCAAUCAAAAGACCUAACCAACAGGACUGUCGUGGUCAUUUAUGCUGUU    60
                                                            M  L  L

  61   GGGGGACAUAGGGGGAAAACAUAUUGCCUUCUUCUACAAGAGGCCUUCAGUCGCCAUAAU   120
        G  D  I  G  G  K  H  I  A  F  F  Y  K  R  P  S  V  A  I  I

 121   UACUUGGCGCCCAAUUUUGGGUUUCAGUUGCUGUUUCCAGCUAUGGGGAGAGGUAAGGUU   180
        T  W  R  P  I  L  G  F  S  C  C  F  Q  L  W  G  E  V  R  L
                                                 M  G  R  G  K  V

 181   AAACCAAACCGUAAAUCGACGGGUGACAAUUCGAAUGUUGUUACUAUGAUUAGAGCUGGA   240
        N  Q  T  V  N  R  R  V  T  I  R  M  L  L  L  *
        K  P  N  R  K  S  T  G  D  N  S  N  V  V  T  M  I  R  A  G

 241   AGCUAUCCUAAGGUCAAUCCGACUCCAACGUGGGUCAGAGCCAUACCUUUCGAAGUGUCA   300
        S  Y  P  K  V  N  P  T  P  T  W  V  R  A  I  P  F  E  V  S

 301   GUUCAAUCUGGUAUUGCUUUUAAAGUACCGGUCGGGUCACUAUUUUCGGCAAAUUUCCGG   360
        V  Q  S  G  I  A  F  K  V  P  V  G  S  L  F  S  A  N  F  R

 361   ACAGAUUCCUUUACAAGCGUCACAGUGAUGAGUGUCCGUGCUUGGACCCAGUUAACACCG   420
        T  D  S  F  T  S  V  T  V  M  S  V  R  A  W  T  Q  L  T  P

 421   CCAGUAAAUGAGUACAGUUUUGUGAGGCUGAAGCCAUUGUUCAAGACUGGUGACUCUACU   480
        P  V  N  E  Y  S  F  V  R  L  K  P  L  F  K  T  G  D  S  T

 481   GAGGAGUUCGAAGGGCGUGCAUCAAACAUCAACACACGAGCUUCUGUAGGGUACAGGAUU   540
        E  E  F  E  G  R  A  S  N  I  N  T  R  A  S  V  G  Y  R  I

 541   CCAACUAAUUUGCGUCAGAAUACUGUGGCAGCCGACAAUGUAUGCGAAGUAAGAAGUAAC   600
        P  T  N  L  R  Q  N  T  V  A  A  D  N  V  C  E  V  R  S  N

 601   UGUCGACAAGUCGCCUUGGUUAUUUCGUGUUGUUUUAACUGAACCUCGACAUAAGCCUUU   660
        C  R  Q  V  A  L  V  I  S  C  C  F  N  *

 661   UGGAUCGAAGGUUAAACGAUCCGCUCCUCGCUUGAGCUUGAGGCGGCGUAUCUCUUAUGU   720

 721   CAACAGAGACACUUUGGUCUAUGGUUGUAUAACAAUAGAUAGACUCCCGUUUGCAAGAUU   780

 781   AGGGUUAACAGAUCUUGCCGUUAGUCUGGUUAGCGCGUAACCGGCCUUGAUUUAUGGAAU   840

 841   AGAUCCAUUGUCCAAUGGCUUUGCCAAUGGAACGCCGACGUGGCUGUAUAAUACGUCGUU   900

 901   GACAAGUACGAAAUCUUGUUAGUGUUUUUCCCUCCACUUAAAUCGAAGGGUUUUGUUUUG   960

 961   GUCUUCCCGAACGCAUACGUUAGUGUGACUACCGUUGUUCGAAACAAGUAAAACAGGAAG  1020

1021   GGGGUUCGAAUCCCUCCCUAACCGCGGGUAAGCGGCCCA  1059
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FIGURE 1

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A

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TMV U5   ------------------------------------------------------

STMV     CCGUUUGCAAGAUUAGGGUUAACAGAUCUUGC---CGUUAGUCUGGUUAG
           ••      ••• •• •  •  •  •••••      ••      ••• ••
TMV U1   UAGUAGAAUUGAUCAGAGGAACCGGAUCUUAUAAUCGGAGCUCUUUCGAG


TMV U5   --------------------------------CACAACUCCGGCUACUUAG
                                          •      •••      •••
STMV     CGCGUAACCGGCCUUGAUUUAUGGAAUAGAUCCAUUGUCCAAUGGCUU--
           ••          •••    • ••• • •• • • •••• •  •••
TMV U1   AGCU--------CUUCUGGUUUGGUUUGGACCUCUGGUCCUGCAACUUGA


TMV U5   CUAUUGUUGUGAGAUUUCCUAAAAUAAAGUCGCUGAAGACUUAAAAUUCA
                       • •• •• •  • •• • ••• •  •• •••  •
STMV     ----------------UGCCAAUGGAACGCCGACGUGGCUGUAUAAUACG
                         ••• •  •• •• •• • ••••
TMV U1   -----GGUAGUCAAGAUGCAUAAUAAAUAACGGAUUGUGUCCGUAAUCAC


TMV U5   GGG-UGGCUGAUACCAAAAUCAGC--AGUGGUUGUUCGUCCACUUAAAUA
           • •• •   •••  •••••    •••• •• • • ••••••••••••
STMV     UCGUUGACAAGUACG-AAAUCUUGUUAGUGUUUUUCCCUCCACUUAAAUC
           •• ••    ••••• • • •  ••••••••••••••••••••••••••
TMV U1   ACG-UGGUGCGUACG-AUAACGCA-UAGUGUUUUUCCCUCCACUUAAAUC


TMV U5   UAACGAUUGUCAUAUCUGGAUCCUACAGUU-AAAC-CAUGUGAUGGUGUA
            •• • •• •  •    ••• •• • •    ••• •••  • • ••••
STMV     GAAGGGUUUUGUUU--UGG-UCUUCCCG----AACGCAUACGUUAGUGUG
           ••••••••• ••• •  ••• ••   • •    •• • ••• • •   •
TMV U1   GAAGGGUUGUGUCU--UGGAUCGCGCGGGUCAAAUGUAUAUGGUUCA-UA


TMV U5   UACUGUGGUAUGG--------CGUAAAACAUCGGAGAGGUUCGAAUCC-U
           •••   •• ••    •••••••        •  ••••••••••••  •
STMV     -ACUACCGU-UGUUCGAAACAAGUAAAACAGGAAGGGGGUUCGAAUCCCU
           •• •••  •        •• •••• • ••    ••••• •••••••••
TMV U1   UACAUCCGCAGG-------CACGUAAUAAAGCGAGGGG-UUCGAAUCCC-


TMV U5   CCCGUAACCGCCGGUA-GCGGCCCA
           ••• ••••••• •••• •••••••
STMV     CCC-UAACCGCGGGUAAGCGGCCCA
           ••• • ••• • •••• • •••••
TMV U1   CCCGUUACCCCCGGUA-GGGGCCCA
```

B   **C**

```
BMV    -GUAAAA--UACCAACUAA                    SPMV  GGGUAUUCCA
         •••••• •••••• ••                            •••
STMV   AGUAAAACUUACCAAUCAA                    STMV  AGUAAAACU
         •••• •••••••                               ••••••
CMV    -GUAA-UCUUACCACUUUC                    STNV  AGUAAAGAC
```

FIGURE 2

3

STRATEGY USED TOWARDS CONSTRUCTION OF A
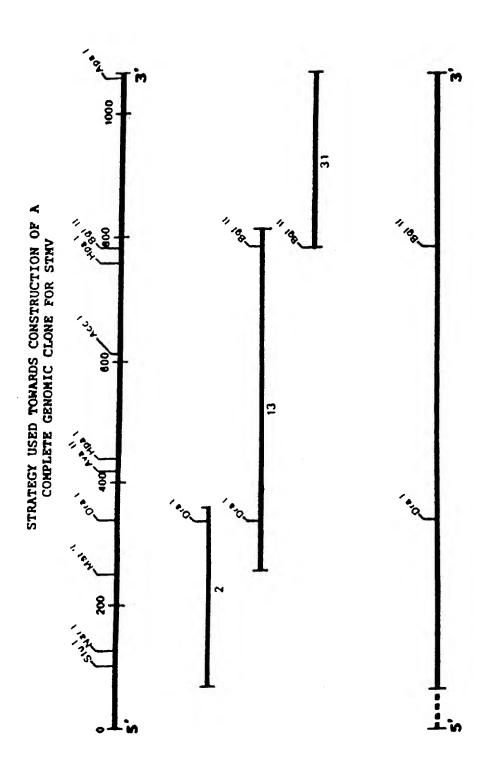COMPLETE GENOMIC CLONE FOR STMV

FIGURE 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01738

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/02; C12N 15/00; C07H 21/04

U.S. CL: 435/70.1,172.3,320; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 4	
Classification System	Classification Symbols
U.S.	435/69.1,70.1,172.3,317.1,320 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

USPTO AUTOMATED PATENT SYSTEM: DIALOG FILES: BIOTECH, PATENTS
See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category*	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X,P Y	Virology (New York USA), Volume 170, issued May 1989, Mirkov et al, "Nucleotide sequence and translation of satellite tobacco mosaic virus RNA," pages 139-146 (see entire document).	1-5 1-29
Y,P	Journal of Cellular Biochemistry (New York, USA) Volume 13 Part D, issued April 1989, Mirkov et al, "Development of satellite tobacco mosaic virus for the expression of heterologous genes in plants," see page 265 (ABSTRACT #M139)	1-29
Y	Plant Molecular Biology Reporter (New York,USA), Volume 1, issued 1983, Van Vloten-Doting, "Advantages of multipartite genomes of single stranded RNA plant viruses in nature, for research, and for genetic engineering," pages 55-60 (see entire document).	1-29
Y	Journal of general virology (Great Britain) , Volume 67, issued 1986, Valverde et al, "Evidence for a satellite RNA associated naturally with the U5 strain and experimentally with the U1 strain of tobacco mosaic virus," pages 1875-1884. (note pages 1879-1881).	22-29
Y	Journal of general virology (Great Britain), Volume 68,issued 1987, Valverde et al, "Same properties of isometric virus particles which contain the satellite RNA of tobacco mosaic virus," pages 965-972 (note page 969 and 972).	1-22

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
15 JUNE 1990	2 6 JUL 1990
International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	P. RHODES NGUYEN NGOC-HO INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (May 1986)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
III. D CUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC ND SHEET)		
Y	Virology (New York, USA), Volume 158, issued 1987, Janda et al, "High Efficiency T7 Polymerase Synthesis of Infectious RNA from Cloned Brome Mosaic Virus cDNA and effects of 5' extensions an transcript infectivity," pages 259-262 (entire document)	1-29
Y	Molecular and Cellular Biology (Washington DC, USA), Volume 4, issued 1984, Ahlquist et al, " cDNA cloning and in vitro transcription of the complete Brome mosaic virus genome," pages 2876-2882 (entire document)	1-29
Y	Proceedings National Academy Sciences USA (Washington DC), Volume 81, issued 1984, Ahlquist et al, " multi-component RNA plant virus infection derived from cloned viral cDNA," pages 7066-7070 (entire document and see page 7070, last paragraph).	1-29
Y	EP, A, 0194809 (Ahlquist et al.) 17 September 1986 (entire document but note especially pages 22-23)	1-29
Y	Science (Washington DC, USA), Volume 231, issued 1986, French et al, "Bacterial gene inserted in an engineered RNA virus: efficient expression in mocotyledanous plant cells," pages 1294-1297. (Note last paragraph).	1-29
Y	Virology (New York, USA), Volume 157, issued 1987, Emmelo et al, "expression in Plants of the cloned satellite tobacco necrosis virus genome and of derived insertion mutants," pages 480-487. (entire document).	1-29
Y	EP, A, 0242016 (BAULCOMBE ET AL.) 21 October 1987 (see entire document).	1-29
Y	EP, A, 0278667 (AHLQUIST ET AL.) 09 February 1987 (see entire document).	1-29

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED TERMS:

STMV, TMV, BMV, vector, RNA virus or viral

Attachment to Form PCT/ISA/210, Part VI.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

I Claims 1-27, drawn to a recombinant RNA and a DNA vector encoding same and to a method of use to transform plant cells; class 435, subclasses 172.1, 172.3, 317.1 and 320.

II Claims 28-29, drawn to a method of use to make protein; class 435, subclass 69.1.